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ESTRUS-INHIBITING SUBSTANCES
IN TESTIS TISSUE EXTRACTS

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY
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By
DAVID ROBARDSON LINCOLN DUNCAN

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Early investigators, both in this country and abroad, using very crude extracts of testis tissue, reported that injection of these crude preparations into normal female rats caused an inhibition of the normal estrous cycle. Interpretations of the mechanism of this inhibition differ according to the worker. Lipschutz¹ and Steinach² considered the inhibition a specific antagonism of male sex hormone on the opposite sex. Moore³ stated that the inhibition was the result of an action of the hormone on the gonadotropic activity of the hypophysis, but that hormones of either sex act on the pituitaries of either sex. Lendle⁴ believed that this inhibitory action did not act through the hypophysis, but directly on the ovary to suppress ovulation. On the basis of this direct action Lendle attempted to use the normal female rat as an assay animal for the male sex hormone. His method, briefly, was to follow the estrous cycles of the rats to make certain that they were regular, then to inject the crude preparations and note the time necessary for a return to the normal cycle as determined by the control period. He chose ten days as an arbitrary length of time for the inhibition of the cycle to last in order to be classed as a positive reaction. While this was obviously a crude qualitative reaction, Lendle claimed that he could show the presence of male hormone in as little as twenty-five grams of fresh testis tissue. However, when lipid extracts of other tissues were injected numerous positive results, inhibition of the cycle, were obtained. From these results we in this laboratory were led to wonder whether the impurities known to be present in the crude extracts were not the causative agents rather than the hormone present in the extracts. With this question in mind we determined to study the different fractions obtained from testis tissue in the preparation of highly purified concentrates of the male hormone.

Preparations.---The method of McGee⁵ as modified by Gallagher and Koch⁶ was used in the preparation of the potent extracts, and is briefly as follows. The testis tissue is ground and extracted with 95 per cent alcohol at room temperature. The alcoholic extract is separated from the tissue residue, and con-

centrated under reduced pressure to a watery sludge. This sludge is very thoroughly extracted with benzene, the insoluble residue discarded, and the extract evaporated to dryness under reduced pressure. The residue from this evaporation is now extracted with acetone. If the extraction with acetone is thorough the residue, composed for the most part of phospholipin, is free of male hormone as measured by the comb-growth method of Koch and Gallagher.⁷ The acetone solution which contains the androgenic activity is evaporated to dryness under reduced pressure and the residue is dissolved in 70 per cent alcohol. This alcoholic solution is shaken thoroughly with hexane which removes from the alcoholic solution the cholesterol and small amounts of phospholipin which are soluble in the acetone due to incomplete anhydrous conditions of extraction. The alcoholic solution, containing the androgenic activity, is evaporated to dryness under reduced pressure, and the residue dissolved in ether. This ether solution is extracted thoroughly and repeatedly with 10 per cent sodium hydroxide in order to remove estrogenic substances. The ether solution of the male hormone is evaporated to dryness and the remaining residue is redissolved in 95 per cent alcohol. This final alcohol solution contains the androgenic activity, and in these studies the purification is not carried any further.

In the preparation of material for this study 227 kilos of fresh bull testes were used, of which 192 kilos were glandular tissue, the remainder being epididymis and tunica vaginalis communis. The benzene extract contained 2095 grams of solid material, the acetone-insoluble fraction weighed 1782 grams, the hexane-soluble fraction contained 296 grams of solids, and the final alcoholic solution, on assay by the comb-growth method, was found to contain between 4400 and 5000 international capon units.

Separation of lecithin and cephalin from crude phospholipin.-- The lecithin and cephalin were separated from the crude phospholipin fraction by the use of cadmium chloride precipitation of the lecithin and subsequent removal of the cadmium chloride with ammonia. This method is the one used by Levene and Rolf⁸ in their studies on lecithin and cephalin. Analysis of the purified lecithin showed it to be 94.8 per cent pure, and the cephalin to be 94.5 per cent pure. These purity figures are based on the relative amounts of amino nitrogen as compared to the total nitro-

gen. The N:P ratio was 1:0.91 for the cephalin and 1:1.05 for the lecithin. Analysis of the crude phospholipin in terms of amino acid total nitrogen showed that the cephalin represented 47.8 per cent of the total.

Method of assay.-- The rats chosen for the assay were virgin female white rats of about three months of age. The estrous cycles were followed by the daily vaginal smear, using the technique of Long and Evans⁹ for about a month and a half. In the rats used the cycles were found to vary from three to nine days. All rats whose cycles did not fall between four and six days in length were discarded. In order to make easier the comparison of the results, all injections were started at the same stage of the cycle. Following the time used by Lendle, injections were started when the vaginal smear showed only squamous epithelial cells. Preliminary studies had shown that the unfractionated crude lipin extract in doses equivalent to 44 grams fresh testis tissue inhibited the cycle. Hence the various fractions described above were administered at the equivalent of 22, 44, 88, 220, and 440 grams fresh testis tissue. Preparations for injection were incorporated in sesame oil or saline, and injections were made twice daily for four days.

Two groups of rats were injected with different doses of the acetone-insoluble fraction, phospholipin, one group receiving 0.406 gram per day, or the equivalent of 44 grams of the fresh testis tissue, the other group receiving 0.203 gram, or the equivalent of 22 grams of the fresh testis tissue. In all the rats the estrous cycle was inhibited, the average for the group receiving the 0.406 gram per day was 15 days, and for the group receiving 0.203 gram the average inhibition was eight days. See Table 1.

Three groups of rats were used to test the hexame-soluble fraction. The first group received 0.135 gram per day, equivalent to 88 grams of fresh testis tissue, the second group received 0.0675 grams per day equivalent to 44 grams of fresh tissue, and the third group received 0.0337 gram per day, equivalent to 22 grams of fresh tissue. Rats receiving 0.135 gram per day showed average inhibition of seven days, rats receiving 0.0675 gram per day showed an average inhibition of five days, and the group receiving 0.0337 gram showed an average inhibition of three days. See Table 1.

Three groups of rats were used for the injections with the non-crystalline male hormone concentrate. One group received 0.00035 gram per day or the equivalent of 44 grams of fresh tissue, the second group received 0.00175 gram per day or the equivalent of 220 grams of fresh tissue, and the third group received 0.0035 gram or the equivalent of 440 grams fresh tissue. Assay of this concentrate by the comb-growth method of Koch and Gallagher⁷ showed that 0.00035 gram was equivalent to 1 international unit. In none of the rats receiving this male hormone concentrate was there the slightest indication of inhibition of the estrous cycle.

Three groups of rats received crystalline testosterone propionate in amounts equivalent to 10, 20, and 40 international units. In the two groups receiving the 10 and 20 I.U. per day there was no sign of cycle inhibition, but the group receiving the 40 I.U. per day showed a definite inhibition of the cycle. On the basis of calculations from the testis tissue concentrate the rats receiving 40 I.U. testosterone propionate per day were given the equivalent of 1760 grams of fresh tissue daily.

From these studies it appears that the results claimed by earlier workers using crude preparations of male hormone might be due to their content of toxic substances and not to their male hormone content. With this idea in mind we examined the different fractions in more detail for the specific inhibitor of the estrous cycle.

A quantity of the hexane-soluble fraction was saponified with a strong alcoholic potassium hydroxide solution for several hours. The cholesterol was separated and recrystallized. A 0.5-gram sample of this cholesterol gave a negative phosphorous test showing that the phospholipin had been removed. On injection of an amount of this purified cholesterol equivalent to 88 grams of fresh tissue (0.135 gram per day), not a single rat showed the slightest inhibition or interference with the cycle. In the previous group of rats injected with the same quantity of impure hexane-soluble fraction the average inhibition of the cycle was seven days. The negative results with cholesterol suggested that the estrous inhibition might be due to phospholipins hence the acetone-insoluble fraction was next investigated.

Lecithin and cephalin were prepared from the crude phos-

pholipin (see Preparations for description of method and analysis). Since the analysis showed that the lecithin and cephalin are present in about equal amounts, the amounts of each one were halved in order to be able to compare the inhibition of each with the inhibition of the crude material. Each rat received 0.203 gram per day or the equivalent of 44 grams of fresh testis tissue. Lecithin, in the amount injected, caused a complete inhibition of the cycle for an average of seven days, and cephalin in like amount caused an inhibition of six days, average. The inhibitions agree very well with those obtained when the crude material was injected.

After observing that both lecithin and cephalin caused inhibition of the cycle we wondered if any constituent part of the lecithin or cephalin molecule would cause an inhibition of the cycle too. In order to try this we obtained synthetic choline chloride and cholamine from the Eastman Kodak Company to be sure that the material would not be contaminated with anything from either the cephalin or the lecithin.

When choline chloride was injected in an amount equivalent to that found in the lecithin equivalent to 44 grams of fresh testis tissue, an interesting thing was noted, namely, that while there was not a total inhibition of the cycle as noted in the other compounds studied, there was a very marked interference with the normal rhythm. To make this statement more clear one needs to recall that in the castrated female rat the vaginal smear is characterized by a complete leucocyte picture at all times while in the normal rat the vaginal smear shows several phases during the cycle. When inhibiting substances are injected the vaginal smear obtained is one in which only leucocytes are present. In the case of the choline-injected animals, the smears contained some nucleated epithelial cells, but the typical squamous epithelial smear, present at one phase of the normal cycle and denoting height of estrus, was not obtained in the rats. Since the rats used for these injections were originally chosen because of the regularity of their cycles, we feel that any great variation from this regular rhythm must be significant. We have chosen to call this variation from the normal an interference with the estrous cycle. In the group of rats injected with 0.0291 gram of choline chloride, equivalent to 44 grams of fresh testis tissue, the average interference was seven days. Cholamine injected in the amount found in the cepha-

lin from 44 grams of fresh tissue, or 0.0064 gram per day, caused an interference in the cycle of six days, average. Finally, when choline chloride and choline were injected in saline solution instead of oil, the same interference was observed but for a shorter period of time, due, no doubt, to the more rapid absorption and excretion. These effects from aqueous choline solutions were more pronounced when the daily dose was spread over four injections instead of two per day. In several of these animals the cycles were completely inhibited.

Finally, lecithins and cephalins prepared from brain, liver, heart, and from hog ovaries also were found to inhibit the estrous cycle in the same way at the levels used for testis-tissue phospholipins.

Discussion

It has been definitely shown that lipid extracts of testis tissue inhibit the estrous cycle of the rat, but the inhibition is not correlated with the androgenic activity of the extract. The best inhibition was obtained with the acetone-insoluble fraction which is known to be free from androgenic activity, while the fraction which contains the greatest androgenic potency gave no estrous inhibitory response in the dosages used. In a study reported by Thrke and D'Amour¹⁰ inhibition was obtained by a particularly purified concentrate. Although their concentrate contained a high concentration of male hormone activity it also contained rather large amounts of phospholipin and it was this phospholipin rather than the hormone which caused the inhibition. This fact can be easily demonstrated by the use of crystalline androgens where the quantity of pure material needed to inhibit the cycle, if expressed in international units of androgen, is over forty times that of the crude phospholipin-containing concentrates. In these studies it was found that 40 I.U. of testosterone propionate were necessary for inhibition, or the equivalent of 1760 grams of fresh testis tissue, as contrasted with 44 grams of fresh testis tissue when the androgen-free, acetone-insoluble material was used. Similar doses of testosterone propionate were found necessary for the inhibition of the estrous cycle in the rat by Browman¹¹ and it is interesting to note that Browman chose these levels of androgen on the assumption that since 20 units of testosterone propionate were not sufficient for the maintenance of normal seminal vesicles in castrated rats it probably would require higher doses to inhibit the pituitary gonadotropic activity. We, therefore, conclude that pure androgens in high doses are capable of inhibiting the estrous cycle and that the reported inhibitions by crude extracts were not due to their androgen content but to other substances. We have shown that the lecithin and cephalin present in the crude phospholipin fraction in equivalent amounts are just as effective in inhibiting the cycle as is the crude material. We have also shown that choline and cholamine, components of lecithin and cephalin, are also capable of interfering with the cycle and in a few

cases actually inhibit the cycle completely. It is entirely possible that if we could reproduce the rate with which the choline is liberated in the body by the hydrolysis of the lecithin, for example by modifying the rate of administration of the pure choline, we might be able to reproduce the complete inhibition of the cycle. This was tried by lowering the individual injection dose and injecting four times daily instead of twice as was the procedure in the earlier work. This in itself did not inhibit the cycle but we were able to get a prolonged interference of the cycle. It is also possible that in the body choline as such is not the most potent substance but it may be that the phosphoric acid ester of choline or the glycerol phosphoric acid ester of choline is the active agent.

The mode and site of action of these inhibiting substances is, of course, of great interest and importance, but no attempt has been made to throw light on this problem. It is possible that the sites of action may be through the pituitary, or directly on the ovary, or vagina, or in both ways. That testosterone propionate can act directly on the vagina without the ovary is clearly shown by its inhibition of estrous response to a definite dose of pure estrone in the spayed rat.¹² Reasoning from this experiment it is not impossible that the materials used in this study acted directly on the end organ, but we have not eliminated the possible effects on the hypophysis or the ovaries which were intact in the test animals.

Summary

These studies include an extension of the earlier reports by us^{13,14} and lead to the following conclusions:

1. The estrus-inhibiting action of crude lipoid extracts from testis tissue is mainly due to the lecithin and cephalin therein.
2. Lecithins and cephalins from brain, liver, heart, and hog ovaries also possess the same property.
3. Choline and cholamine also interfere with the estrous cycle in the rat.
4. Pure crystalline testosterone propionate also inhibits the estrous cycle in the rat, but it requires at least 40 international units of androgen.
5. Many non-androgenic substances probably interfere with the estrous cycle.

TABLE 1

Substance or fraction	Amount injected	Weight equiva- lent of fresh tissue	Minimum inhibi- tion	Maximum inhibi- tion	Average inhibi- tion	Number of animals
	g./cc.	g.	days	days	days	
Acetone- insoluble	0.406	44	12	18	15	15
Acetone- insoluble	0.203	22	7	10	8	15
Hexane- soluble	0.135	88	6	10	7	15
Hexane- soluble	0.0675	44	3	7	5	15
Hexane- soluble	0.0337	22	2	4	3	15
Hexane- soluble (purified)	0.135	88	0	0	0	15
Non-crys- talline male hormone con- centrate	0.00035*	44	0	0	0	10
Non-crys- talline male hormone con- centrate	0.00175	220	0	0	0	10
Non-crys- talline male hormone con- centrate	0.00350***	440	0	0	0	10
Lecithin	0.203	44	5	9	7	5
Cephalin	0.203	44	4	8	6	5
Choline (in oil)	0.0291	44	4**	9**	7**	5
Cholamine (in oil)	0.0064	44	2**	8**	6**	5
Choline (aqueous)	0.0291	44	2**	6**	4**	5
Cholamine (aqueous)	0.0064	44	2**	6**	4**	5
Testosterone propionate	0.00015***	440	0	0	0	5
Testosterone propionate	0.0003	880	0	0	0	5

TABLE 1 -- Continued

Substance or fraction	Amount injected	Weight equiva- lent of fresh tissue	Minimum inhibi- tion	Maximum inhibi- tion	Average inhibi- tion	Number of animals
	g./cc.	g.	days	days	days	
Testosterone propionate	0.0006	1760	3	7	6	10

* or 1 international unit.

** denotes interference with the estrous cycle rather than inhibition.

*** or 10 international units.

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